AD_____

IMMUNOLOGIC CONTROL BY ORAL VACCINES OF DIARRHEAL DISEASE DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI AND SHIGELLA

ANNUAL / FINAL REPORT

Report prepared by:
Myron M. Levine, M.D., D.T.P.H.
Principal Investigator

June 30, 1988

Supported by:
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21701-5012

Contract No. DAMD 17-83-C-3074
DAMD 17-78-C-8011

Center for Vaccine Development,
Department of Medicine,
University of Maryland School of Medicine
Baltimore, MD 21201

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Center for Vaccine Development Department of Medicine 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) University of Maryland Baltimore, MD 21701-5012	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-83-C-3074 DAMD17-78-C-8011
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 64758A; PROJECT NO. 3S4-64758D849; TASK NO. AA; WORK UNIT ACCESSION NO. 071

11. TITLE (Include Security Classification) (U) Immunologic Control by Oral Vaccines of Diarrheal Disease Due to Enterotoxigenic Escherichia coli and Shigella

12. PERSONAL AUTHOR(S)
Myron M. Levine

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 12/1/77 TO 8/31/87	1988 June 30	50

16. SUPPLEMENTARY NOTATION
Annual Report covers period January 1, 1986 - August 31, 1987.

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
RA 1, Enterotoxigenic E. coli; diarrheal disease; CFA/I, CFA/II; Immunization; Salmonella; Shigella; oral vaccine; Genetically engineered vaccine; enteropathogenic E. coli.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Travelers' diarrhea in several different clinical forms represents an important source of morbidity and loss of efficiency among United States Military personnel deployed in less-developed areas of the world. The single most common recognized etiologic agent of travelers' diarrhea is enterotoxigenic Escherichia coli, while the major cause of the dysenteric form of travelers' diarrhea (i.e. accompanied by diarrheal stools with blood and mucus) is Shigella. Research carried out under this contract was aimed at developing safe and effective immunizing agents to prevent these diarrheal infections of military importance.

Candidate oral vaccines against ETEC that were evaluated included purified CS1 and CS3 colonization factor fimbriae and a prototype attenuated strain that expresses CS1 and CS3 fimbriae but does not elaborate LT or ST toxins. The live oral vaccine gave the best secretory IgA antifimbrial antibody response.

Studies with Shigella included carrying out a dose-response with pathogenic S. sonnei to establish a model to assess the efficacy of candidate Shigella vaccines. Strain 5076-1C,

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301-663-7325
	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

Block 19 continued:

a modification of attenuated Salmonella typhi strain Ty21a into which is introduced the 120 Md plasmid of S. sonnei, was found to be safe and immunogenic as a live oral Shigella vaccine. Some lots of vaccine provided significant protection against experimental challenge, while others did not. Vaccine candidate 7931-1-2-9, consisting of an E. coli K-12 containing chromosomal genes for expression of the type and group antigens of S. flexneri 2a and the 140 Md flexneri invasiveness plasmid was evaluated for safety, immunogneicity and efficacy. This vaccine caused some adverse reactions at a dose of 10^9 organisms. Recipients of lower, non-reactogenic doses were not protected against experimental challenge with pathogenic S. flexneri 2a.

FOREWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

BACKGROUND

Diarrheal diseases represent one of the principal causes of morbidity and loss of efficiency among military personnel deployed in less-developed areas of the world (1-8). Measures must be developed to diminish the incidence of and loss of productivity due to travelers' diarrhea in military populations. The most common recognized agent of travelers' diarrhea in U.S. adults visiting or working in less-developed countries is enterotoxigenic Escherichia coli (ETEC) (9-14). Shigella, the major causative agent of bacillary dysentery, is often the second or third most frequently isolated agent in studies of the etiology of travelers' diarrhea (9,13). Although the incidence of diarrheal illness due to Shigella is much lower than due to ETEC, the severity of the average case is much greater, so that a soldier is lost from his duties for a longer period of time. Furthermore, Shigella infections can be transmitted by direct contact involving very low infective inocula (as few as 10 Shigella organisms can cause clinical illness (15). ETEC infections, in contrast, require much larger inocula (circa 10^6 organisms) and are typically transmitted by contaminated food and water vehicles (16). Thus, both ETEC and Shigella represent infectious agents of considerable military importance.

ENTEROTOXIGENIC E. COLI

The Center for Vaccine Development of the University of Maryland School of Medicine has been involved in a long-term program to develop immunizing agents to prevent ETEC diarrhea. This program has involved studies of the pathogenesis of ETEC diarrhea, of the human immune response and the evaluation of

certain prototype vaccine candidates. Studies carried out under Research Contract DAMD17-78-C-8011 paved the way for studies performed under Research Contract DAMD17-83-C-3074. Under Research Contract DAMD17-78-C-8011 a volunteer model of experimental enterotoxigenic E. coli diarrhea was established (17). This allowed studies to assess the importance of specific virulence properties and the first studies to assess whether immunity occurs following clinical infection with ETEC. In the mid-1970s, for example, there was great skepticism expressed by microbiologists, epidemiologists and infectious disease consultants as to whether ETEC strains that express only the heat-stable (ST) but not the heat-labile (LT) enterotoxin were pathogenic for humans. Levine et al (18) fed to volunteers an ETEC strain, 214-4, that elaborates only ST. This strain, which had been originally isolated from a traveler with diarrhea, caused clear-cut diarrhea in volunteers, demonstrating that ST-only ETEC strains can indeed be pathogenic. Also in the mid-1970s, fimbrial colonization factors were identified among human ETEC pathogens. The first such fimbrial antigen, colonization factor antigen I (CFA/I) was described by Evans et al (19,20). Later Evans et al (21) described CFA/II. These investigators implied that all human ETEC strains must possess these fimbrial colonization factors, in order to be pathogenic. Levine et al (22) examined a series of ETEC challenge strains known to cause diarrhea in volunteers and showed that several of the strains did not express CFA/I or II yet were pathogenic. Levine et al hypothesized that other antigenically distinct colonization factors must exist among human ETEC pathogens

besides CFA/I and II; this was subsequently proven to be correct (23-25).

Under Research Contract DAMD17-78-C-8011, Levine et al showed that clinical infection with an ETEC strain that elaborates both LT and ST, strain B7A (O148:H28), provided significant protection against re-challenge nine weeks later with the same organism (17). Diarrhea occurred in only 1 of 8 re-challenged volunteers but in 7 of 12 controls ($p=0.05$). This homologous re-challenge study was the first demonstration of infection-derived immunity to ETEC and represented a hallmark observation to help direct the development of vaccines against ETEC. It was notable that although the re-challenged volunteers were clinically protected they shed the ETEC challenge strain with the same frequency as control volunteers. This suggested that the mechanism of protection against ETEC did not involve gut bactericidal mechanisms. Levine et al hypothesized that intestinal secretory IgA (SIgA) directed aginst relevant ETEC antigens was the mediator of protection. The two most likely antigens hypothesized as being involved were LT and fimbrial colonization factors. A subsequent challenge study was designed to determine whether infection-derived anti-LT by itself could protect. Volunteers who convalesced from diarrhea due to LT/ST ETEC strain B7A (O148:H28) were re-challenged several weeks later with a strain of a different O:H serotype, E2528C1 (O25:NM), that elaborates only LT. Despite the fact that the volunteers had manifested a serological response to LT after illness with B7A, they were not protected against challenge with E2528C1. The results of this clinical research study suggested

that anti-LT by itself may not be protective. As a consequence, subsequent research focused on studies of anti-colonization factor immunity. Anti-colonization immunity was envisioned to involve SIgA antibodies directed against surface antigens of the bacteria concerned with colonization of the proximal small intestine, the critical anatomic site of the host/bacteria interaction that leads to diarrhea. This would prevent ETEC from attaching to receptors on enterocytes of the proximal intestine. The bacteria would then be swept into the large intestine, still in a viable state, where they would proliferate unimpeded in the lumen. This hypothesis could explain the results of the volunteer studies described in Reference 17.

Virtually all ETEC strains express type 1 somatic fimbriae (22,26), as do the vast majority of normal intestinal flora E. coli. In this sense there is no evidence that type 1 somatic fimbriae are a virulence property of ETEC. Type 1 somatic fimbriae are characterized by their ability to hemagglutinate guinea pig erythrocytes, a property that is inhibited by D-mannose. It is likely that the function of type 1 fimbriae is to anchor E. coli to mucus in the large intestine, allowing colonization of that anatomic site by the E. coli. It was suggested by Brinton et al (27) that stimulation of an immune response to type 1 somatic fimbriae might nevertheless provide protection against colonization of the proximal small intestine by ETEC. Brinton et al purified type 1 somatic fimbriae of strain H10407 to be used as a parenteral vaccine. This vaccine was shown to be well-tolerated and to elicit both serum IgG and intestinal IgA responses against type 1 somatic fimbriae (28,29). In one of three challenge studies volunteers

were protected against challenge with pathogenic strain H10407; it was deemed likely, however, that anti-O78 antibody stimulated by contaminating LPS in the fimbrial vaccine probably mediated the protection seen in that one study. When imunized volunteers were challenged with an ETEC strain of a distinct O:H serotype that expressed type 1 somatic fimbriae antigenically closely related to those of H10407, no protection was seen. The conclusion drawn from these studies was that a vaccine based on stimulating an immune response to type 1 somatic fimbriae is not of great value in protecting against ETEC.

ETEC is an important pathogen causing diarrhea among neonatal and infant herd animals, including calves and piglets. The causative strains are of different O:H serotypes from those that cause human disease. The fimbrial colonization factors present in the animal strains are also antigenically distinct; they include K88, K99, F41 and 987-pili. Vaccines consisting of purified K88, K99 or 987 fimbriae were used by veterinary investigators to immunize pregnant sows and cows (30-33). Newborn piglets and calves suckled on immunized mothers were significantly protected against challenge with otherwise lethal doses of ETEC strains expressing the homologous fimbriae. Immunity was not cross-protective. These impressive results in veterinary studies stimulated studies of vaccines in man intended to stimulate anti-colonization factor immunity.

In order for ETEC to cause disease in man they must be able to colonize the small intestine, overcoming the usually potent peristaltic defense mechanism (34). Fimbrial colonization factors

which bind the ETEC to receptors on enterocytes of the small intestine protect the bacteria from the effects of peristalsis. Under Research Contract DAMD17-83-C-3074 we sought to identify the fimbrial colonization factors associated human ETEC, to study the serum and intestinal SIgA immune response to these fimbrial antigens and to evaluate candidate vaccines intended to elicit anti-fimbrial immunity.

Fimbrial Colonization Factors among ETEC Strains in Relation to Toxin Phenotype

Levine et al (26,35) examined a series of ETEC strains from patients with travelers' diarrhea isolated in various parts of the world for the presence of the fimbrial colonization factors recognized at that time including CFA/I and CFA/II. CFA/I or CFA/II was identified in 7 of 10 LT/ST strains but in only 2 of 26 LT-alone or ST-alone strains.

Identification of New Fimbrial Colonization Factors of ETEC

It was obvious that there must exist additional fimbrial colonization factors other than CFA I and CFA II because these antigens were never associated with ETEC strains of certain common O serogroups isolated from diverse geographic sources, including O27, O115, O148, O159 and O167 (34). Investigators at the Central Public Health Laboratory, Colindale, England reported the existence of antigenically distinct fimbrial antigens in ETEC strains of serogroups O25, O27, O115, O148 and O167 (23). This new antigen was originally referred to as PCF E8775. Subsequently it was shown that E8775 represents a family of three distinct antigens that were

referred to as CS4, CS5 and CS6 (36,37). CS6 is common to virtually all the E8775 ETEC strains but often in conjunction with CS4 or CS5. CS4 and CS5 are rigid fimbriae circa 6-7 nm in diameter. The morphology of CS6 has not yet been described.

The last major ETEC serogroup not to be associated with a known fimbrial antigen was O159, particularly of the common serotype O159:H4. Investigators at the Center for Vaccine Development identified an antigenically distinct fimbrial colonization factor antigen in O159:H4 strains, which they referred to as PCF O159:H4 (25).

Antigenic Heterogeneity in Strains Expressing CFA/II

When Evans et al (19,20) reported the existence of a plasmid-mediated fimbrial colonization factor (CFA/I) in LT/ST strains of serogroups O78, O25 and O63, other investigators confirmed this observation and exchanges of prototype strains and anti-CFA/I antisera among different laboratories throughout the world demonstrated that there was no antigenic heterogeneity within CFA/I of diverse sources. Evans et al had originally identified CFA/I on the basis of the property whereby strains expressing this factor hemagglutinate human type A and bovine erythrocytes despite the presence of D-mannose (which prevents the hemagglutinating properties of type 1 somatic fimbriae).

Evans et al (21) subsequently described another fimbrial colonization factor, which they referred to as CFA II. CFA II was reported to confer on ETEC strains the property of mannose-resistant hemagglutination of bovine erythrocytes and was

found to be associated with ETEC strains of serogroups O6 and O8. However, when other investigators attempted to corroborate the findings of Evans et al and when reference strains were exchanged, much confusion occurred as discrepancies were found to exist. A major step towards resolution of this confusing situation was made when Cravioto et al (38) reported that antigenic heterogeneity existed within CFA/II. These investigators discovered that so-called CFA/II was not a single antigen but was rather a family of three distinct antigens; some ETEC strains expressed only one of the antigens, while others concomitantly expressed two of the antigens. Cravioto et al referred to these three distinct antigens as components 1, 2 and 3. Independently, Smyth (39) also discovered that CFA/II comprised a family of three separate antigens, which he referred to as Coli Surface antigens 1, 2 and 3 (CS1, CS2 and CS3). Smyth reported that virtually all strains bearing CFA/II express CS3; in addition many also express CS1 or CS2 in conjunction. No strains were found which express CS1 and CS2 simultaneously. Furthermore, Smyth showed that which CS antigens are expressed is a function of the serotype and biotype of the ETEC strain. Smyth (39) showed that CS1 and CS2 antigens were fimbrial in nature and consisted of rigid structures 6-7 nm in diameter. In contrast, the morphology of CS3, the antigen common to virtually all "CFA/II" strains was not ascertained. Similarly, investigators at the Central Public Health Laboratory, Colindale, England described CS3 as being non-fimbrial and amorphous (40,41).

Under contract DAMD17-83-C-3074, we were investigating anti-colonization factor immunity, particularly among CFA/II

strains. Thus, it became imperative to study more intensively the characteristics of CS3, the common antigen of the CFA/II family. Levine et al (42) purified CS3 and CS1 to homogeneity and prepared antisera against these antigens. The specificity of the antisera was demonstrated in immunoblotting studies utilizing purified CS1 and CS3. Levine et al (42) confirmed that CS1 consisted of rigid fimbriae with a diameter of 6-7 nm. However, for the first time the morphology of CS3 was also described and was found to consist of flexible, wiry, fibrillar type of fimbriae with a diameter of 2-3 nm. Other fibrillar type fimbriae include K88 and F41 found in animal ETEC isolates. By means of gold-immunolabelling electron microscopy, Levine et al (42) confirmed that the fibrillar fimbrial structures that they visualized were indeed CS3.

Animal Studies with Purified CS1/CS3 Combination Vaccine

Studies were carried out in rabbits to determine whether enteral immunization with purified CS1/CS3 fimbriae vaccine would inadvertantly elicit immunological tolerance to fimbriated organisms inoculated parenterally. This was a safety concern expressed by one of the Human Volunteer Commitees that reviewed the proposed clinical protocols (29). The basis for the concern was the observation that in some species certain antigens administered orally induce tolerance to those antigens when they are presented paenterally (43-45). Since some vaccinees might at some later time in life have to mount immune responses to E. coli in the course of bacteremia secondary to urinary tract infection, cholecystitis, etc., it was considered prudent to carry out animal studies. As

shown in Levine et al (29), rabbits immunized enterally into chronic Thiry-Vella intestinal loops, mounted brisk SIgA antifimbrial antibody responses; nevertheless, these rabbits responded following parenteral inoculation with fimbriated E. coli bearing the homologous fimbriae.

Rabbits were given multiple oral doses of purified CS1/CS3 fimbriae vaccine, with $NaHCO_3$ and cimetidine; a total of 1.4 mg was given to each rabbit. Approximately one month after completion of immunization, the rabbits were challenged with pathogenic ETEC of strain E24377A by the RITARD technique (reversible intestinal tie acute rabbit diarrhea) model. There was no difference in attack rate between the immunized and the control rabbits (46).

Clinical Studies with Purified CS1/CS3 Fimbriae

A group of volunteers received multiple oral doses of purified CS1/CS3 fimbriae vaccine prepared from strain M424-C1 (O6:H16, biotype A) (77,78). In order to protect the protein vaccine from the possible deleterious effects of gastric acid, volunteers received cimetidine several hours before ingesting vaccine, in order to diminish gastric acid production, while vaccine was administered concomitantly with $NaHCO_3$ to neutralize gastric acid. Only 2 of 10 vaccinees manifested a significant rise in either serum IgG or intestinal SIgA antibody to CS1 or CS3 fimbriae (Table 1). These volunteers participated in a challenge study to assess vaccine efficacy. Not surprisingly, there was no evidence of vaccine efficacy elicited by the fimbrial vaccine (Table 2). These studies are summarized in Levine et al (46).

It was not clear why the CS1/CS3 purified fimbrial vaccine elicited such a poor response in humans when it had stimulated prominent SIgA anti-fimbrial antibody responses when applied to the mucosa of chronic Thiry-Vella loops of rabbits (29). Investigators in the Department of Gastroenterolcgy at the Walter Reed Army Institute of Research showed that the antigenicity of purified CS1/CS3 vaccine was adversely affected by gastric contents even at pH 7.0 (48). Presumably this is due to the effects of proteolytic enzymes that are having demonstrable, albeit diminished, activity even at neutral pH. To confirm the hypothesis that gastric contents adversely affect the purified CS1/CS3 fimbriae, we immunized a group of volunteers with purified CS1/CS3 vaccine by direct intraduodenal inoculation via intestinal tube and collected intestinal fluid on several occasions post-inoculation to measure antibody. Under these circumstances, significant rises in SIgA anti-fimbrial antibody were detected in 4 of 5 vaccinees, versus only 2 of 10 who received oral vaccine ($p=0.046$, Fisher's Exact test, 1 tail) (Table 3). It became apparent from these studies that if purified fimbriae are to be used as an oral vaccine they will have to be delivered by means of enteric-coated capsules or some other mechanism that completely protects the vaccine protein from exposure to gastric contents.

Clinical Studies with a Prototype Live Oral Vaccine against ETEC

E1392-75-2A is an O6:H16 biotype A isolate that expresses CS1 and CS3 fimbrial antigens but does not elaborate LT or ST and fails to hybridize with LT and ST gene probes. This spontaneous

laboratory mutant, derived from an LT/ST strain, was evaluated in volunteers for safety, immunogenicity and efficacy as a live oral vaccine. The characteristics of strain E1392-75-2A are summarized in Levine et al (29). In initial clinical studies it was found that a single dose of E1392-75-2A given with buffer stimulated brisk serum and intestinal SIgA responses in the majority of vaccinees, including all who received doses $\geq 10^{10}$ organisms. The vaccine was generally well-tolerated. However, reminiscent of what has been encountered in recipients of some live oral cholera vaccines (49,50), approximately 10% of the volunteers who ingested E1392-75-2A developed some loose stools (29).

In order to explore the magnitude and kinetics of the immune response elicited by E1392-75-2A and whether anti-fimbrial immunity is protective, a group of volunteers were given a single oral 5 x 10^{10} organism dose of the prototype vaccine strain. A strong serum and intestinal SIgA antibody response to CS1 and CS3 fimbriae was recorded (Table 4). Moreover, the geometric mean titer of gut SIgA anti-fimbrial antibody (416) was approximately 10-fold higher than that noted in volunteers immunized with three 5 mg doses of purified CS1/CS3 vaccine given intraduodenally via intestinal tube. These data show that live vaccines interact with the host intestinal immune system in a manner both qualitatively and quantitatively different from inactivated vaccines.

A group of volunteers who received a single oral dose (5 x 10^{10} organisms) of E1392-75-2A were challenged one month later with 10^{8} organisms of pathogenic strain E24377A; the latter strain elaborates LT and ST and CS1 and CS3 but is of a different

serotype (O139:H28). Thus if E1392-75-2A were to show protection against challenge with E24377A it would be on the basis of anti-fimbrial immunity. As shown in Table 5, only 3 of 12 vaccinees developed diarrhea versus 6 0f 6 controls ($p<0.005$, 75% vaccine efficacy). Moreover, as shown in Table 6, bacteriological studies demonstrated that the mechanism of protection was by preventing E24377A organisms from colonizing the proximal small intestine. The proportion of individuals excreting the challenge organism and the mean level of excreton was the same in vaccinees and controls. However, only 1 0f 12 vaccinees tested had positive duodenal cultures versus 5 of 6 controls ($p<0.004$). Thus in the vaccinees anti-colonization immunity was evident and was correlated with SIgA antibody to CS1 and CS3 fimbriae (Table 4). As a consequence of these studies, we have undertaken to develop strains of E. coli that express fimbrial colonization factors and B subunit of LT but do not elaborate biologically active LT or ST. It is hoped that such strains will colonize the intestine and stimulate potent immune responses without causing adverse reactions.

Studies of the LT Antitoxin Response

A somewhat surprising revelation made in the early 1980s was that there were antigenic differences between the LT elaborated by porcine ETEc strains and that of human ETEC strains (51-53). Indeed, antigenically, the difference was about as great as that between cholera toxin and either LT. Consequent to these reported antigenic differences in human LT (LTh) and porcine LT (LTp), we undertook to assess the immune response to LTh, LTp and cholera toxin in persons infected with ETEC or with cholera. These studies

resulted in methods for the specific serodiagnosis of ETEC and cholera infection and provided helpful aid for seroepidemiological studies (54). These studies are summarized in Levine et al (54).

ENTEROPATHOGENIC E. COLI

Enteropathogenic E. coli (EPEC) represent another major category of diarrheagenic E. coli (34,55). EPEC belong to certain classical O:H serotypes, do not elaborate LT or ST and do not manifest Shigella-like enteroinvasiveness. Studies of the pathogenesis of EPEC diarrhea have greatly enhanced our knowledge of novel mechanisms by which E. coli cause diarrhea.

It was discovered that most EPEC of classical serotypes adhere to HEp-2 cells in tissue culture by the formation of microcolonies, giving rise to a pattern of adherence referred to as localized adherence (LA) (56-59). This was shown to be a property mediated by a plasmid circa 60 Md in size, the so-called EPEC adherence factor (EAF) plasmid (57-59). Transfer of the plasmid to E. coli HB 101 was accompanied by transfer of the LA property, while loss of the EAF plasmid from an EPEC strain was followed by loss of the ability to manifest LA (57). A 1 kb fragment of the EAF plasmid has been shown to function as a sensitive and specific DNA probe to identify EPEC (60,61).

Studies in volunteers have shown that the 60 Md EAF plasmid is necessary for expression of the full pathogenicity of EPEC strains (62). The plasmid has been shown to encode the production of a 94 Kd protein against which humans mount a serological response (62). This outer membrane protein is a candidate antigen for future vaccines against EPEC.

SHIGELLA

Inactivated Shigella organisms utilized as parenteral whole cell vaccines have failed to protect monkeys or man in experimental challenge studies or humans in controlled field trials (63-65), despite stimulating high titers of circulating antibody. In contrast, some attenuated strains of Shigella used as live oral vaccines have been safe and protective in volunteer challenges and in controlled field trials (66-70). In particular, the streptomycin-dependent (SmD) Shigella strains of Mel et al (67-69) and the T_{32} S. flexneri 2a strain of Istrati et al (71) have shown considerable promise as safe and protective vaccines (67-72). Nevertheless, these attenuated strains have a number of drawbacks that encourage research to prepare improved attenuated strains. Both the SmD and the T_{32} strains represent mutants with undefined genetic lesions responsible for the attenuation; as a consequence, it is theoretically possible for these strains to undergo genetic reversion to a virulent state. Indeed, streptomycin-independent revertants of the SmD S. sonnei vaccine strain occurred with some lots (73), although there was no evidence that these particular revertants were capable of causing disease. The SmD vaccines required four doses to provide primary immunization and annual boosters to maintain protection (69). An ideal live oral Shigella vaccine would require fewer doses. The SmD vaccines caused vomiting in a small percentage of children after the first dose of vaccine. Ideally, a Shigella vaccine should not be associated with any adverse reactions. A prototype vaccine created by Dr. S.B. Formal and coworkers at the Walter Reed

Army Institute of Research in the mid 1970s consisted of an E. coli K-12 expressing the group and type specific antigens of S. flexneri 2a. This vaccine was well-tolerated but colonized the intestine poorly, elicited weak immune responses and did not provide significant protection in experimental challenge studies in volunteers (74).

Because of the above-mentioned shortcomings of the earlier live oral Shigella vaccines, Formal et al at WRAIR produced two new prototype Shigella vaccines. The first prototype consisted of attenuated Salmonella typhi strain Ty21a into which was introduced the 120 Md invasiveness plasmid of S. sonnei. This hybrid strain, 5076-1C, expresses the O antigens of both S. sonnei and S. typhi (75). The second prototype oral Shigella vaccine prepared at WRAIR in the mid 1980s consists of E. coli K-12 into which was introduced the chromosomal genes encoding the group and type specific O antigens as well as the invasiveness plasmid S. flexneri 2a (76). Results of clinical studies evaluating the safety, intestinal colonizing potential, immunogenicity and efficacy of these vaccine candidates are presented below.

Strain 5076-1C, S. typhi-S. sonnei Bivalent Oral Vaccine

Strain 5076-1C was grown on solid agar and lyophilized at the Forest Glen Vaccine Production Facility of WRAIR. Three separate clinical studies were carried out with lots # 2 and # 5, involving vaccination of volunteers with three 2×10^9 organism doses given on an every other day schedule. Approximately one month after completion of vaccination the immunized volunteers and a similar number of controls were challenged with 5×10^2 pathogenic S. sonnei (76). Results of these three vaccination/challenge studies

are presented in detail in Black et al (76). The vaccination was very well-tolerated and provided significant protection against diarrhea and dysentery. Based on the safety and efficacy of 5076-1C vaccine found with lots # 2 and # 5, a large lot of 5076-1C vaccine was prepared at Forest Glen with a sufficient number of doses to carry out field trials of efficacy in Israel, Chile and Thailand. However, this lot of vaccine, lot # 8, failed to confer significant protection in volunteers against experimental challenge. Furthermore, two separate lots of 5076-1C vaccine lyophilized at the Swiss Serum and Vaccine Institute after growth in liquid broth failed to provide significant protection of volunteers in experimental challenge studies of vaccine efficacy.

E. coli/S. flexneri 2a Vaccine Strain 7931-1-2-9

Strain 7931-1-2-9 designates the E. coli K-12 that expresses S. flexneri 2a O antigen and possesses the flexneri invasiveness plasmid; this strain invades HeLa cells. In step-wise fashion involving three cohorts of volunteers, strain 7931-2-9 was fed to a total of 17 individuals in doses of 5×10^6 (2 volunteers), 5×10^7 (2 volunteers) or 10^9 (13 volunteers). As summarized in Table 7, four vaccinees manifested adverse reactions including two who had fever, one who had mild diarrhea and a fourth who experienced a single small dysenteric stool with blood and mucus. The serological responses of the vaccinees are also summarized in Table 7. As with previous live oral Shigella vaccines, rises in serum O antibody were seen in approximately only one-fourth of vaccinees. Notably, the only individual who manifested rises in serum O antibody to both O antigen and to the

invasiveness-associated outer membrane proteins was the individual who experienced diarrhea during vaccination; this individual also had a rise in intestinal SIgA antibody to O antigen.

Since all the adverse reaction had occurred in recipients of the 10^9 organism dose of vaccine and lower doses of vaccine appeared to be well-tolerated (albeit involving small numbers of vaccinees), the next group of volunteers were given three 5 x 10^6 organism doses of vaccine with buffer. No adverse reactions were recorded among these 13 vaccinees (Table 8). Accordingly, a challenge study was planned to assess vaccine efficacy. As a preliminary, a dose response was carried out with pathogenic S. flexneri 2a to determine the appropriate dose for the challenge model. Eight volunteers ingested 10^2 and eight received 10^3 pathogenic S. flexneri 2a organisms of strain 2457T. Results, summarized in Table 9, show that 4 of 8 individuals who ingested 10^2 organisms manifested clinical illness; in three individuals this involved diarrhea, fever and dysentery. At a dose of 10^3 organisms of pathogenic strain 2457T, the attack rate for clinical illness was 5 of 8. A dose of 10^2 was selected for the challenge study to assess vaccine efficacy.

Eight volunteers who had received three 5 x 10^6 organism doses of 7931-2-9 vaccine were challenged (approximately one month after completion of vaccination) with 10^2 pathogenic S. flexneri 2a, along with 8 unimmuinized control volunteers. Also challenged at the same time were a volunteer who had ingested a single 5 x 10^7 organism dose of vaccine and another who had received a single 10^9 organism dose. Results of this challenge study,

summarized in Table 10, were disappointing. There was no evidence of vaccine efficacy. Indeed, 4 of 10 vaccinees developed clinical illness, of whom all four had dysentery and three had fever. The results of these studies show that if the modified E. coli K-12 approach to preparing Shigella vaccine candidates is to be further pursued, the next candidates must be less reactogenic and more immunogenic. There is some expectation that with appropriate genetic modifications this may be achievable.

TABLE 1

Summary of Serum and Intestinal Fluid Antibody Response In Volunteers Immunized with Oral CFA/II (CS1, CS3) Vaccine*

	CS1 & CS3 Fimbrial Antigens
Significant Rises in Serum IgG ELISA Antibody	2/10+
Singificant Rises in Intestinal SIgA ELISA Antibody	2/10

*1.7 mg twice weekly for 4 weeks. Volunteers took cimetidine 3 hours before ingesting vaccine with $NaHCO_3$.

+No. with significant seroconversion/No. vaccinees tested.

TABLE 2

Clinical Response Following Challenge of Immunized (8 Oral Doses of CFA/II Pili Vaccine) Volunteers and Controls with Enterotoxigenic *E. coli* Strain E23477A (0139:H28, CS1,CS3)

	Diarrheal Attack Rate	Mean Diarrheal Stool Volume Per Ill Volunteer	Mean No. Loose Stools Per Ill Volunt
Controls	6/9*	576 ml (442-782)**	5 (3-7)
Vaccinees	3/8	847 (510-1290)	5.3 (2.7)

*No. ill/No. volunteers challenged.

**(Range).

TABLE 3

Immunogenicity of Purified CFA/II (CS1, CS3) Fimbriae Administered Orally or Enterally

Method of Vaccination	Significant Rises (>4-Fold) in SIgA Intestinal Fluid Antibody to CFA/II (CS1,CS3)	
Oral*	2/10**	p=0.046++
Enteral+	4/5**	

*1.7 mg doses twice weekly for four weeks (total 13.6 mg); vaccine given after cimetidine and $NaHCO_3$ treatment.

+5 mg doses on day 1, 14, and 28.

**No. seroconverters/No. vaccinated.

++Fisher's Exact Test, single tail.

TABLE 4

Immunogenicity of Live Oral Non-Enterotoxigenic *E. coli* Vaccine Bearing CFA/II Fimbriae E1392/75-2A

Antigen	Significant Rises	Geometric Mean Titer Pre-Vaccination	Post Vaccination	Pre-Challenge
CFA/II (CS1, CS3)	10/10	5	416	315
06	10/10	6	91	165
0139	0/10	2	2	2
LT	0/10	2	2	2

TABLE 5

Efficacy of a Single Dose of Live Oral E. Coli Vaccine E1392-75-2A (06:H16,CS1,CS3) in Protecting Against Diarrhea Following Challenge with $5X10^9$ E. coli E24377A (0139:H28, LT^+/ST^+, CS1,CS3)

Group	Diarrheal Attack Rate		Severity of Diarrhea Per Ill Volunteer: Mean No. Stools	Mean Stool Volume
Controls	6/6*		8.8 (2-18)+	1147 ml (315-1855)
		p<.005		
Vaccinees	3/12		3.7 (2.5)	713 (229-1110)

*No. ill/No. challenged.

+(Range)

TABLE 6

Bacteriologic Findings in E1392-75-2A Vaccinees and Controls Following Challenge with Enterotoxigenic *E. coli* Strain E24377A (0139:H28, LT^+/ST^+, CS1, CS3)

Group	Duodenal Cultures		Stool
Controls	5/6* ($7X10^3$)+		6/6 ($8X10^8$)
		p<.004	
Vaccinees	1/2 (10^1)		12/12 ($1X10^8$)

*No. Positive/No. volunteers challenged.

+(Mean no. *E. coli* 24377A per gm stool or duodenal fluid.)

TABLE 7

SHIGELLA CVD 2002 - CLINICAL, MICROBIOLOGIC AND IMMUNOLOGIC RESPONSE TO VACCINATION WITH E. COLI/S. FLEXNERI 2A VACCINE STRAIN 7931-1-2-9

STUDY AND VOLUNTEER NUMBER	DOSE	CLINICAL RESPONSE TO VACCINATION: DIARRHEA	DYSENTERY	FEVER	VACCINE STRAIN EXCRETION IN STOOL: DURATION (DAYS)	IMMUNOLOGY - 4-FOLD RI: SERUM LPS(IgA)	SERUM LPS(IgG)	SERUM + PCP(IgG)	BY ELISA JEJUNAL FLUID LPS(sIgA)
2002 -17	$(5 \times 10^6) \times 3$	-	-	-	9	-	-	- *	NT
-18	$(5 \times 10^6) \times 3$	-	-	-	7	-	-	- *	-
-8	$(5 \times 10^7) \times 3$	-	-	-	12	-	-	- *	+
-12	$(5 \times 10^7) \times 3$	-	-	-	7	-	-	-	+
-2	10^9	-	-	101[4b]	4	+	-	+	-
-3	10^9	-	-	-	5	-	-	-	-
-7	10^9	435 ML[c]	-	-	3	-	-	- *	-
-9	10^9	-	-	-	4	-	-	+	-
-10	10^9	80 ML[d]	+	-	4	-	-	- *	-
-11	10^9	-	-	-	4	-	-	+	-
-13	10^9	-	-	-	4	+	-	-	+
-14	10^9	-	-	-	4	+	-	+	-
-15	10^9	-	-	-	4	-	-	-	NT
-16	10^9	-	-	100[e]	3	-	+	-	+
-19	10^9	-	-	-	4	-	-	+	-
-20	10^9	-	-	-	4	-	-	-	-
-21	10^9	-	-	-	-	-	-	-	-
TOTAL ADVERSE REACTIONS:			4/17			3/17	1/17	5/17	4/15

b 27 HOURS AFTER VACCINATION, ALSO HAD MALAISE AND HEADACHE
c 83 HOURS AFTER VACCINATION
d 1 SMALL DYSENTERIC STOOL 43 HOURS AFTER VACCINATION.
e 27 HOURS AFTER VACCINATION, ALSO HAD GROSSLY BLOODY GRADE 2 STOOL, MALAISE, HEADACHE, ABDOMINAL CRAMPS
* HAD POLYPEPTIDE RESPONSES BY WESTERN BLOT IN PRE AND POST IMMUNIZATION SERA
+ PCP = PLASMID CODED POLYPEPTIDE
NT = NOT TESTED

TABLE 8

SHIGELLA CVD 2003 - CLINICAL, MICROBIOLOGIC AND IMMUNOLOGIC RESPONSE TO VACCINATION WITH E. COLI/S. FLEXNERI 2A VACCINE STRAIN 7931-1-2-9

STUDY AND VOLUNTEER NUMBER	** DOSE	CLINICAL RESPONSE TO VACCINATION DIARRHEA	DYSENTERY	FEVER	VACCINE STRAIN EXCRETION IN STOOL DURATION (DAYS)	IMMUNOLOGY - 4-FOLD RISES BY ELISA SERUM LPS(IgA)	SERUM LPS(IgG)	SERUM + PCP(IgG)	JEJUNAL FLUID LPS(sIgA)
2003 -1	10^7	-	-	-	1	-	-	-	-
-2	10^7	-	-	-	7	-	-	-	-
-4	10^7	-	-	-	8	-	-	+	-
-5	10^7	-	-	-	7	-	-	-	-
-6	10^7	-	-	-	7	-	-	-	-
-7	10^7	-	-	-	10	-	-	-	NT
-8	10^7	-	-	-	5	-	-	-	NT
-9	10^7	-	-	-	6	-	-	-*	--
-10	10^7	-	-	-	11	-	+	+*	+
-11	10^7	-	-	-	9	-	-	-*	-
-12	10^7	-	-	-	11	-	-	-	-
-13	10^7	-	-	-	7	-	-	-	-
-14	10^7	-	-	-	8	-	-	-*	NT
TOTAL ADVERSE REACTIONS:		1/13					1/13	2/13	1/10
TOTAL (2002 AND 2003):		5/30				3/30	2/30	7/30	5/25

* HAD POLYPEPTIDE RESPONSES BY WESTERN BLOT IN PRE AND POST IMMUNIZATION SERA

\+ PCP = PLASMID CODED POLYPEPTIDE

** ACTUAL INOCULUM 5 X 10 CFU

NT = NOT TESTED

Table 9

SHIGELLA CVD 2001

DOSE RESPONSE CHALLENGE STUDY

SHIGELLA FLEXNERI 2a 2457T

Volunteer	Mean no. of loose stools	Diarrheal stool volume	Dysentery	Peak fever	Coprocultures
Group A - 10^2:					
2001-1	-	-	-	-	-
-2	38	2069	+	103^2	+
-3	-	-	-	-	+
-4	-	-	+	-	+
-5	-	-	-	-	-
-6	15	1178	+	103^2	+
-8	30	1525	+	102^8	+
-9	-	-	-	-	-
Group B - 10^3:					
-10	-	-	-	-	+
-11	12	518	+	100^7	+
-12	5	424	-	-	+
-13	-	-	-	-	+
-14	34	1354	+	102^1	+
-15	13	884	+	101^3	+
-17	12	3028	+	104	+
-18	-	-	-	-	-

Table 10

SHIGELLA CVD 2004
CLINICAL AND MICROBIOLOGICAL RESPONSE TO CHALLENGE WITH S. FLEXNERI 2A STRAIN 2457T (300 CFU) IN RECIPIENTS OF E. COLI/S. FLEXNERI VACCINE 7931-1-2-9 VERSUS CONTROLS

VACCINEES		DIARRHEA	TOTAL NUMBER OF DIARRHEA STOOLS	DYSENTERY	FEVER	SHIGELLA STOOL EXCRETION (DAYS)
2004	-2	575	6	+	103^{8}	3
	-3	1885	15	+	104^{4}	2
	-5	227	7	+	-	5
	-6	-		-	-	9
	-8	-		-	-	-
	-9	-		-	-	-
	-12	548	6	+	100^{2}	>3
	-14	-		-	-	-
	-17	-		-	-	-
	-18	-		-	-	-
CONTROLS						
2004	-1	383	4	+	101^{4}	8
	-4	-		-	-	-
	-7	278	3	+	102^{4}	4
	-11	-		-	-	-
	-13	73	2	+	100^{8}	9
	-15	-		-	-	5
	-16	-		+ *	-	3
	-19	676	6	+	100^{1}	6

* SINGLE GRADE 2 STOOL

NOTE: VACCINEE #6 RECEIVED SINGLE DOSE OF 10^{9} CFU OF VACCINE IN SHIGELLA CVD 2002 STUDY (VOLUNTEER #2002 -2)

VACCINEE #8 RECEIVED 3 DOSES OF 5 X 10^{7} CFU OF VACCINE IN SHIGELLA CVD 2002 STUDY (VOLUNTEER #2002 -8)

REMAINING VACCINEES RECEIVED 3 DOSES OF 5 X 10^{6} CFU OF VACCINE IN SHIGELLA CVD 2003 STUDY

REFERENCES

1. Philbrook RR, Gordon JE. 1958. Diarrhea and dysentery. Chapter XVII. In: Preventive Medicine in World War II. Coates JB Jr, Hoff EC, Hoff PM, eds. Office of the Surgeon General, Washington, D.C.

2. Griffin RB, Jr., Gaines S. Diarrhea in a U.S. battle group in Thailand. Milit Med 129:546-550, 1964.

3. Gilbert DN, Greenberg JH. Vietnam: Preventive medicine orientation. Milit Med 132:769-790, 1967.

4. Forman DW, Tong MJ, Murrell, Cross JH. Etiology study of diarrheal disease in Vietnam. Am J Trop Med Hyg 20:598-601, 1971.

5. Echevarria P, Childres PV, Anderson G, Cross JH. Heat-labile enterotoxigenic Escherichia coli infections in new arrivals at Subic Naval Base, the Philippines. Milit Med 144:173-174, 1979,

6. Bulmer E. A survey of tropical diseases as seen in the Middle East. Roy Soc Trop Med Hyg 37:225-242, 1944.

7. Rowe B, Taylor J, Bettelheim K. An investigation of travelers' diarrhoea. Lancet I:1-5, 1970.

8. Rowe B. Escherichia coli O148 and diarrhoea in adults. Brit Med J. 3:741, 1970.

9. DuPont Hl, Olarte J, Evans DG, Pickering L, Galindo E, Evans DJ. Comparative susceptibility of Latin American and United States students to enteric pathogens. N Eng J Med 295:1520-1521, 1976.

10. Echevarria P, Blacklow NR, Sanford LB, Cukor GG. Traveler's diarrhea among American peace Corps volunteers in rural Thailand. J Infect Dis 143:767-771, 1981.

11. Santosham M, Sack RB, Froelich J, Yolken R, Kapikian A, Javier C, Medina C, Orskov F. Biweekly prophylactic doxycycline for travelers' diarrhea. J Infect Dis 143:598-601, 1981.

12. Sack DA, Kaminsky DC, Sack RB, Itotia JN, Authur RR, Kapikian AZ, Orskov F, Orskov I. Prophylactic doxycycline dor travelers diarrhea. Results of prospective double-blind study of Peace Corps volunteers in Kenya. N Eng J Med 298:757-763, 1978.

13. Black RE. Pathogens that cause travelers' diarrhea in Latin America and Africa. Rev Infect Dis 8 supplement:S131-S135,1986.

14. Taylor DN, Ecchavarria P. Etiology and epidemiology of travelers' diarrhea in Asia. 8 supplement:S136-S141, 1986.

15. Levine MM, Dupont HL, Formal SB, Hornick RB, Takeuchi A, Gangarosa EJ, Snyder MJ, Libonati JP. Pathogenesis of Shigella dysenteriae-1 (Shiga) dysentery. J Infect Dis 127:261-270, 1973.

16. Levine MM, Rennels MB, Cisneros L, Hughes TP, Nalin DR, Young CR. Lack of person-to person transmission of enterotoxigenic Escherichia coli despite clcse contact. Am J Epidemiol 111:347-355, 1980.

17. Levine MM, Nalin DR, Hoover DL, Bergquist EJ, Hornick RB, Young CR. Immunity to enterotoxigenic Escherichia coli. Infect Immun 23:729-736, 1979.

18. Levine MM, Caplan ES, Waterman D, Cash RA, Hornick RB, Snyder MJ. Diarrhea Due to Escherichia coli that produce only heat-stable enterotoxin. Infect Immun 17:78-82, 1977.

19. Evans DG, Siulver RP, Evans DJ Jr, Chase DG, Gorbach SL. Plasmid-controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic for humans. Infect Immun 12:656-667, 1975.

20. Evans DG, Evans DJ Jr, Tjoa WS, DuPont HL. Detection and characterization of colonization factor of enterotoxigenic Escherichia coli isolated from adults with diarrhea. Infect Immun 19:727-736, 1978.

21. Evans DG, Evans DJ Jr. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic Escherichia coli of serogroups O6 and O8. Infect Immun 21:638-647, 1978.

22. Levine MM, Rennels MB, Daya V, Hughes TP. Hemagglutination and colonization factors in enterotoxigenic and enteropathogenic Escherichia coli that cause diarrhea. J. Infect. Dis. 141:733-737, 1980.

23. Thomas LV, Cravioto A, Scotland SM, Rowe B. New fimbrial antigenic type (E8775) that may represent a colonization factor in enterotoxigenic Escherichia coli in humans. Infect Immun 35:1119-1124, 1982.

24. Honda T, Arita M, Miwatani T. Characterization of new hydrophobic pili of human enterotoxigenic Escherichia coli: a possible new colonization factor. Infect Immun 43:959-965, 1984.

25. Tacket CO, Maneval DR, Levine MM. Purification, morphology, and genetics of a new fimbrial putative colonization factor of enterotoxigenic Escherichia coli O159:H4. Infect Immun 55:1063-1069, 1987.

26. Levine MM, Ristaino P, Sack RB, Kaper JB, Orskov F, Orskov I. Colonization Factor Antigens I and II and type 1 somatic pili in enterotoxigenic Escherichia coli: relation to enterotoxin type. Infect. Immun. 39:889-897, 1983.

27. Brinton CC Jr. The piliation phase syndrome and the uses of purified pili in disease control. Proceedings of the 13th Joint U.S.-Japan conference on cholera. Atlanta, Ga., September, 1977. Department of HEW publication no. 178-1590. NIH, Bethesda, Md, 1977.

28. Levine MM, Black RE, Brinton CC Jr, Clements ML, Fusco P, Hughes TP, O'Donnell S, Robins-Browne R, Wood S, Young CR. Reactogenicity, immunogenicity and efficacy studies of Escherichia coli type 1 somatic pili parenteral vaccine in man. Scand J Infect Dis supplement. 33: 83-95, 1982.

29. Levine MM, Black RE, Clements ML, Young CR, Cheney CP, Schad P, Collins H, Boedeker EC, Prevention of Enterotoxigenic Escherichia coli Diarrheal Infection in Man by Vaccines that Stimulate Antiadhesion (Anti-Pili) Immunity. In: Boedecker EC (ed.), Attachment of Organisms to the Gut Mucosa. Boca Raton: CRC Press, pp. 223-244, 1984.

30. Acres SD, Isaacson R, Babiuk L, Kapitary RA. Immunization of calves against enterotoxigenic colibacillosis by vaccinating dams with purified K99 antigen and whole cell bacteria. Infect Immun 25:121-126, 1979.

31. Morgan RL, Isaacson R, Moon H, Brinton CC, To CC. Immunization of suckling pigs against enterotoxigenic Escherichia coli induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge. Infect Immun 22:771-777, 1978.

32. Nagy B, Moon HW, Isaacson R, To CC, Brinton CC. Immunization of suckling pigs against enteric enterotoxigenic Escherichia coli infection by vaccinating dams with purified pili. Infect Immun 21:269-274, 1978.

33. Rutter JM, Jones G. Protection against enteric disease cuased by Escherichia coli - a model for vaccination with a virulence determinant. Nature 242:531-532, 1973.

34. Levine MM. Escherichia coli that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent. J Infect Dis 155:377-380, 1987.

35. Levine MM, Adhesion of Enterotoxigenic Escherichia coli in Man and Animals. In: Taylor-Robinson D, (ed.), Adhesion and Pathogenicity. Ciba Symposium: pp.142-154, 1981.

36. McConnell MM, Thomas LV, Day NP, Rowe B. Enzyme-linked immunosorbent assays for the detection of adhesion factor antigens of enterotoxigenic Escherichia coli. J Infect Dis 152:1120-1127, 1985.

37. Thomas LV, McConnell MM, Rowe B, Field Am. The possession of three novel coli surface antigens by enterotoxigenic Escherichia coli positive for the putative colonisation factor CFA/I CF8775. J Gen Microbiol 131:2319-2326, 1985.

38. Cravioto A, Scotland S, Rowe B. Hemagglutination activity and colonization factor antigens I and II in enterotoxigenic and non-enterotoxigenic Escherichia coli isolated from humans. Infect Immun 36:189-197, 1982.

39. Smyth C. Two mannose-resistant haemagglutinins on enterotoxigenic Escherichia coli of serotype O6:K15:H16 or H- isolated from travelers and infantile diarrhea. J Gen Microbiol 128:2081-2096, 1982.

40. Smith HR, Scotland S, Rowe B. Plasmids that code for production of colonisation factor antigen II and enterotoxin production in strains of Escherichia coli. Infect Immun 40:1236-1239, 1983.

41. Mullany P, Field AM, McConnell MM, Scotland SM, Smith HR, Rowe B. Expression of plasmids coding for colonization factor antigen II (CFA/II) and enterotoxin production in Escherichia coli. J Gen Microbiol 129:3591-3601, 1983.

42. Levine MM, Ristaino P, Marley G, Smyth C, Knutton S, Boedeker E, Black R, Young C, Clements ML, Cheney C, Patnaik R. Coli Surface antigens 1 and 3 of colonization factor antigen II-positive enterotoxigenic Escherichia coli: morphology, purification, and immune responses in humans. Infect Immun 44:409-420, 1984.

43. Tomasi TB Jr. Oral tolerance. Transplantation. 29:353, 1980.

44. Swarbrick ET, Stokes CR, Soothill JF. Absorption of antigens after oral immunization and the simultaneous induction of specific systemic tolerance. Gut20:121- , 1979,

45. Challacombe SJ, Tomasi TB Jr. Systemic tolerance and secretory immunity after oral immunization. J Exp Med 152:1459-146 , 1980.

46. Levine MM, Morris JG, Losonsky G, Boedeker E, Rowe B, Fimbriae (pili) Adhesins as Vaccines. In: Lark D, Normak S, Brent-Uhlin E, Eds., Protein-Carbohydrate Interactions in Biological Systems. London: Academic Press, pp. 143-145, 1986.

47. Levine MM, Black RE, Clements ML, Morris JG, Losonsky G, Boedeker E, Rowe B. Fimbriae (pili) adhesins as vaccines. In: Abstracts of the 21st Joint Conference on Cholera. U.S.-Japan Cooperative Medical Science Program, 1985, bethesda, Md.

48. Schmidt M, Kelley L, Tseng Y, Boedeker EC. Towards an oral E. coli pilus vaccine for travelers diarrhea: susceptibility to proteolytic digestion. Gastroenterology :1575, 1985.

49. Levine MM, Black RE, Clements ML, Lanata C, Sears S, Honda T, Young CR, Finkelstein RA. Evaluation in man of attenuated Vibrio cholerae El Tor Ogawa strain Texas Star-SR as a live oral vaccine. Infect Immun 43:515-522, 1984.

50. Levine MM, Kaper JB, Herrington D, et al: Volunteer studies of deletion mutants of Vibrio cholerae O1 prepared by recombinant techniques. Infect Immun 56:161-167, 1988.

51. Geary S, Marchlewicz BA, Finkelstein RA. Comparison of heat-labile enterotoxins from porcine and human strains of Escherichia coli. Infect Immun 36:215-220, 1982.

52. Honda T, Tsuji T, Takeda Y, Miwatani T. Immunological nonidentity of heat-labile enterotoxins from human and porcine enterotoxigenic Escherichia coli. Infect Immun 34:337-340, 1981.

53. Takeda Y, Honda T, Sima H, Tsuji T, Miwatani T. Analysis of antigenic determinants in cholera enterotoxin and heat-labile enterotoxins from human and porcine enterotoxigenic Escherichia coli. Infect Immun 41:50-53, 1983.

54. Levine MM, Young CR, Black RE, Takeda Y, Finkelstein RA. Enzyme-lined immunosorbent assay to measure antibodies to purified heat-labile enterotoxins from human and porcine strains of Escherichia coli and to cholera toxin: application in serodiagnosis and seroepidemiology. J Clin Microbiol 21:174-179, 1985.

55. Levine MM, Edelman R. Enteropathogenic Escherichia coli of classical serotypes associated with infant diarrhea-epidemiology and pathogenesis. Epidemiol Rev 6:31-51, 1984.

56. Cravioto A, Gross RJ, Scotland SM, Rowe B. An adhesive factor found in strains of Escherichia coli belonging to the traditional enteropathogenic serotypes. Curr Microbiol 3: 95:99, 1979.

57. Baldini MM, Kaper JB, Levine MM, Candy DCA, Moon HW. Plasmid-mediated factors adhesion in enteropathogenic Escherichia coli. J Pediatr Gastroenterol Nutr 2:534-538, 1983.

58. Nataro JP, Scaletsky ICA, Kaper JB, Levine MM, Trabulsi LR. Plasmid mediated factors conferring diffuse and localized adherence of enteropathogenic Escherichia coli. Infect Immun 48:378-383, 1985.

59. Nataro JP, Kaper JB, Robins-Browne R, Prado V., Vial PA, Levine MM Patterns of adherence of diarrheagenic Escherichia coli to HEp-2 cells. Ped Infect Dis J 6:829-831, 1987.

60. Nataro JP, Baldini MM, Kaper JB, Black RE, Levine MM. Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J Infect Dis 152:560-565, 1985.

61. Levine MM, Prado V, Robins-Browne RM, Lior H, Kaper JB, Moseley S, Gicquelais K, Nataro JP, Vial P, Tall B. DNA probes and HEp-2 cell adherence assay to detect diarrheagenic Escherichia coli. J Infect Dis 158:in press, 1988.

62. Levine MM, Nataro JP, Baldini MM, Kaper JB, Black RE, Clements ML, O'Brien AD. The Diarrheal response of humans to some classical serotype enteropathogenic Escherichia coli is dependent on a plasmid encoding an enteroadhesiveness factor. J Infect Dis 152:550-559, 1985.

63. Formal SB, Maenza RM, Austin S, LaBrec EH. Failure of parenteral vaccines to protect against experimental shigellosis. Proc Soc Exp Biol Med 125:347-349, 1967.

64. Shaughnessy HJ, Olsson RC, Bass K, Friewer F, Levinson SO. Experimental human bacillary dysentery: polyvalent dysentery vaccine. in its prevention. J Am Med Ass 132:362-368, 1946.

65. Higgins AR, Floyd TM, Kader MA. Studies in shigellosis. III. A controlled evaluation of a monovalent Shigella vaccine in a highly endemic environment. Am J Trop Med Hyg 4:281-288, 1955.

66. DuPont HL, Hornick RB, Snyder MJ, Libonati JP, Formal SB, Gangarosa EJ. Immunity in shigellosis. II. Protection induced by oral live vaccine or primary infection. J Infect Dis 125:12-16, 1972.

67. Mel DM, Terzin AL, Vuksic L. Studies on vaccination against bacillary dysentery. 3. Effective oral immunization against Shigella flexneri 2a in a field trial. Bull WHO 32:647-655, 1965.

68. Mel DM, Arsic BL, Nikolic BD, Radovanovic ML. Studies on vaccination against bacillary dysentery. 4. Oral immunization with live monotypic and combined vaccines. Bull WHO 39:375-380, 1968.

69. Mel DM, Arsic BL, Radovanovic ML, Litvinjenko S. Live oral Shigella vaccine: vaccination schedule and the effect of booster dose. Acta Microbiol Acad Sci Hung 21:109-114, 1974.

70. Levine MM, Gangarosa EJ, Barrow WB, Weiss CF. Shigellosis in custodial institutions. V. Effect of intervention with streptomycin-dependent Shigella sonnei vaccine in an institution with endemic disease. Am. J. Epidemiol. 104:88-92, 1976.

71. Istrati G, Meitert T, Ciufecu C. Recherches sur l'immunite active de l'homme dans la dysenterie bacillaire. Arch Roum Pathol Exp Microbiol 24:677-686, 1965.

72. Wang Bingrui. Study on the effect of oral immunization of T32-Istrati strain against bacillary dysentery in field trials. Arch Roum Pathol Exp Microbiol 43:285-290, 1984.

73. Levine MM, Gangarosa EJ, Barrow WB, Norris GK, Wells JG, Weiss CF. Shigellosis in Custodial Institutions. IV. In Vivo stability and transmissibility of oral attenuated streptomycin-dependent Shigella vaccines. J Infect Dis 131:704-707, 1975.

74. Levine MM, Woodward WE, Formal SB, Gemski P, Jr., DuPont HL, Hornick RB, Snyder MJ. Studies with a new generation of oral attenuated Shigella vaccine: Escherichia coli bearing Shigella surface antigens. J Infect Dis 136:577-582, 1977.

75. Formal SB, Baron L, Kopecko D, Washingtom O, Powell C, Life C. Construction of a potential bivalent vaccine strain: introduction of Shigella sonnei form I antigen genes into the galE Salmonella typhi Ty21a typhoid vaccine. Infect Immun 34:746-750, 1981.

76. Black RE, Levine MM, Clemnts ML, Losonsky G, Herrington H, Berman S, Formal SB. Prevention of shigellosis by a Salmonella typhi-Shigella sonnei bivalent vaccine. J Infect Dis 155:1260-1265, 1987.

CONTRACT-RELATED PUBLICATIONS

Contract DAMD 17-83-C-3074

1. Levine MM, Ristaino P, Marley G, Smyth C, Knutton S, Boedeker E, Black R, Young C, Clements ML, Cheney C, Patnaik R. Coli Surface Antigens 1 and 3 of Colonization Factor Antigen II-Positive Enterotoxigenic Escherichia coli: Morphology, Purification, and Immune Responses in Humans. Infect. Immun. 44:409-420, 1984.

2. Levine MM, Young CR, Black RE, Takeda Y, Finkelstein RA. Enzyme-lined Immunosorbent Assay to Measure Antibodies to Purified Heat-Labile Enterotoxins from Human and Porcine Strains of Escherichia coli and to Cholera Toxin: Application in Serodiagnosis and Seroepidemiology. J. Clin. Microbiol. 21:174-179, 1985.

3. Levine MM, Nataro JP, Baldini MM, Kaper JB, Black RE, Clements ML, O'Brien AD. The Diarheal Response of Humans to Some Classical Serotype Enteropathogenic Escherichia coli is Dependent on a Plasmid Encoding an Enteroadhesiveness Factor. J. Infect. Dis. 152:550-559, 1985.

4. Black RE, Levine MM, Clements ML, Losonsky G, Herrington D, Berman S, Formal SB. Prevention of Shigellosis by a Salmonella typhi-shigella sonnei Bivalent Vaccine. J. Infect. Dis. 155:1260-1265, 1987.

5. Tacket CO, Maneval DR, Levine MM. Purification, Morphology, and Genetics of a New Fimbrial Putative Colonization Factor of Enterotoxigenic Escherichia coli 0159:H4. Infect. Immun. 55:1063-1069, 1987.

6. Levine MM, Xu J-G, Kaper JB, Lior H, Prado V, Tall B, Nataro J, Karch H, Wachsmuth IK. A DNA Probe to Identify Enterhemorrhagic Escherichia coli 0157:H7 and Other Serotypes that Cause Hemorrhagic Colitis and Hemolytic Uremic Syndrome. J. Infect. Dis. 156:156:175-182, 1987.

7. Karch H, Heesemann J, Laufs R, Kroll H-P, Kaper JB, Levine MM. Serological response to type 1-like somatic fimbriae in diarrheal infection due to classical enteropathogenic Escherichia coli. Micro. Patho. 2:425-434, 1987.

8. Karch H, Heeseman J, Laufs R, O'Brien AD, Tacket CO, Levine MM. A Plasmid of Enterohemorrhagic Escherichia coli 0157:H7 is Required for Expression of a New Fimbrial Antigen and for Adhesion to Epithelial Cells. Infect. Immun. 55:455-461, 1987.

9. Kaper JB, Levine MM. Progress towards a vaccine against enterotoxigenic Escherichia coli. Vaccines. 6:197-199, 1988.

Contract Related Publications

Contract DAMD 17-78-C-8011

1. Levine MM, Nalin DR, Hoover DL, Bergquist EJ, Hornick RB, Young CR. Immunity to Enterotoxigenic Escherichia coli. Infect. Immun. 23:729-736, 1979.

2. Levine MM, Rennels MB, Daya V, Hughes TP. Hemagglutination and Colonization Factors in Enterotoxigenic and Enteropathogenic Escherichia coli that Cause Diarrhea. J. Infect. Dis. 141:733-737, 1980.

3. Levine MM, Rennels MB, Cisneros L, Hughes TP, Nalin DR, Young CR. Lack of Person-to-Person Transmission of Enterotoxigenic Escherichia coli Despite Intimate Contact. Am. J. Epidemiol. 111:347-355, 1980.

4. Clements ML, Levine MM, Black RE, Robins-Browne RM, Cisneros LA, Drusano GL, Lanata CF, Saah AJ. Lactobacillus Prophylaxis for Diarrhea Due to Enterotoxigenic Escherichia coli. Antimicrob. Ag. Chemother. 20:104-108, 1981.

5. Robins-Browne R, Levine MM. Effect of Chlorpromazine on Intestinal Secretion Mediated by Escherichia coli Heat-Stable Enterotoxin and 8-Br-Cyclic GMP in Mice. Gastroenterology. 80:321-326, 1981.

6. Robins-Browne RM, Levine MM, Rowe B, Gabriel EM. Failure to Detect Conventional Enterotoxin in Classical Enteropathogenic (serotyped) Escherichia coli Strains of Proven Pathogenicity. Infect. Immun. 38: 798-801, 1982.

7. Black RE, Levine MM, Clements ML, Cisneros L, Daya V. Treatment of Experimentally Induced Enterotoxigenic Escherichia coli Diarrhea with Trimethoprim, Trimethoprim/Sulfamethoxazole, or Placebo. Rev. Infect. Dis. 4:550-545, 1982.

8. Candy DCA, Leuang TSM, Mak RHK, Harries JT, Marshall WC, Phillips AD, Robins-Browne R, Chadwick MV, Levine MM. A Fimbrial Antigen Mediating Adhesion and Hemagglutination of Escherichia coli from Children. FEMS Microbiol. Lett. 15:325-329, 1982.

9. Levine MM, Black RE, Brinton CC Jr, Clements ML, Fusco P, Hughes TP, O'Donnell S, Robins-Browne R, Wood S, Young CR. Reactogenicity, immunogenicity and efficacy studies of Escherichia coli type 1 somatic pill parenteral vaccine in man. Scand. J.Infect. Dis. Supplement. 33: 83-95, 1982.

10. Levine MM. Vaccines to Prevent Travelers' Diarrhea. Therapeutische Umschau. 40:253-256, 1983.

11. Somatic Pili in Enterotoxigenic Escherichia coli: Relation to Enterotoxin Type. Infect. Immun. 39:889-897, 1983.

12. Levine MM, Traveller's Diarrhoea: Prospects for Successful Immunoprophylaxis. Scand. J. Gastroenterol. Vol. 18 supplement 84, p. 121-134, 1983.

13. Baldini MM, Kaper JB, Levine MM, Candy DCA, Moon HW. Plasmid Mediated Adhesion in Enteropathogenic Escherichia coli. J. Ped. Gastroenterol. 2:534-538, 1983.

14. Ristaino P, Levine MM, Young CR. An Improved GM_1-Enzyme-Linked Immunosorbent Assay for the detection of Escherichia coli Heat-Labile Enterotoxin. J. Clin. Microbiol. 18:808-815, 1983.

15. Levine MM, Black RE, Clements ML, Young CR, Cheney CP, Schad P, Collins H, Boedeker EC, Prevention of Enterotoxigenic Escherichia coli Diarrheal Infection in Man by Vaccines that Stimulate Antiadhesion (Anti-Pili) Immunity. In: Boedecker EC (ed.), Attachment of Organisms to the Gut Mucosa. Boca Raton: CRC Press, pp. 223-244, 1984.

16. Lanata CF, Kaper JB, Baldini MM, Black RE, Levine MM. Sensitivity and Specificity of DNA probes for the Detection of E. coli Enterotoxins Using the Stool Blot Techniques. J. Infect. Dis. 152:1087-1090, 1985.

17. Levine MM, Jorris JG, Losonsky G, Boedeker E, Rowe B. Fimbriae (Pili) Adhesins as Vaccines. In: Lark DL (ed.), The Molecular Biology of Microbial Pathogenicity. London: Academic Press, pp. 143-145, 1986.

18. Black RE, Levine MM, Clements ML, Hughes T, O'Donnell S. Association Between O Blood Group and Occurrence and Severity of Diarrhoea Due to Escherichia coli. Trans. Royal Soc. Trop. Med. Hyg. 81:120-123, 1987.

DISTRIBUTION LIST

1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
5 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-PLE Fort Detrick, Frederick, Maryland 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100